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Optimization of the PCR for Detection of *Staphylococcus aureus nuc* Gene in Bovine Milk

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ABSTRACT

Staphylococcus aureus is an economically important and a major mastitis-causing pathogen that also poses food safety and antimicrobial resistance threats. Substances in mastitic milk inhibit the *Taq* DNA polymerase reaction (*Taq* PCR) making it of limited use for detecting *S. aureus* mastitis. In the study reported here, a set of oligonucleotide primers of 21 and 24 bases was used in *Taq*-PCR to amplify DNA from *S. aureus* (isolates from bovine mastitis). A specific amplicon of 270 bp was generated as predicted. Replacing *Taq* DNA polymerase with *Thermus thermophilus* (*Tth*) DNA polymerase alone (*Tth*-PCR) raised the sensitivity of *S. aureus* detection in milk from experimentally infected cows from 65 to 80%. Combining the use of *Tth* DNA polymerase and the purification of crude DNA extract using Chelex-100 before PCR raised the sensitivity to 100%. In a random survey involving 100 milk samples from cattle not infected with *S. aureus*, the test was 100% specific. With milk samples from clinical cases of bovine mastitis, 100% sensitivity and specificity were also observed. It is concluded that *Tth*-PCR on milk samples with the purification of crude DNA extracts using Chelex-100 is as sensitive as but faster than conventional milk bacteriological culture techniques and is highly specific. The modified PCR correlates with elevated somatic cell counts, detects evidence of chronic and resolving infection based on *S. aureus*-specific DNA and circumvents the endogenous inhibitory effects of milk.
(**Key words:** polymerase chain reaction, inhibitors, *Staphylococcus aureus*, mastitis)

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Abbreviation key: *Taq*-PCR = *Taq* DNA PCR, ***Tth*-PCR** = *Tth*-DNA PCR, **UIUC** = University of Illinois at Urbana-Champaign.

INTRODUCTION

PCR has proven to be an extremely powerful research tool (27). However, its full potential as a diagnostic method in disease remains to be proven. A major limitation of PCR-based diagnostic tests is the inhibition of DNA polymerase reaction with (***Taq* PCR**) by many substances found in clinical material (31). Blood, mucus, urine, sperm, vitreous fluids, and fecal material are some of the body fluids and products that have been shown to inhibit *Taq* DNA polymerase (31, 40). In addition, a variety of other substances may have inhibitory effects on *Taq* DNA polymerase activity (16, 40). Accordingly, the application of PCR for diagnostic purposes largely depends on the methods by which *Taq* DNA polymerase inhibitory materials can be either removed or destroyed, or on the replacement of *Taq* DNA polymerase by an enzyme that is not vulnerable to these inhibitory materials.

Attempts to avoid *Taq* DNA polymerase inhibitory materials in clinical specimens have included stringent washing of the specimen, dilution of the specimen, heparinase treatment of heparinized blood, and heating of target DNA prior to PCR (3, 10, 20, 24, 26). While these modifications raise the sensitivity of PCR, none of them completely removes the inhibitory materials from the specimen. In addition, the exact methods tend to be applicable to only one kind of specimen.

Methods of extracting DNA from a variety of biological materials have included separation and purification steps utilizing phenol-chloroform extractions or ethanol precipitation or both (5, 23). Inorganic extraction procedures have included the use of high salt concentration (8) and excess proteinase K digestion (15) and the use of glass powder (36). Although these methods are suc-

cessful in recovering high molecular weight DNA from large samples, they require several steps and may include transfers of DNA extracts to additional containers or washing and desalting of filters or columns. These additional steps allow increased opportunities for cross-transfer of samples or the introduction of contaminants (37). Also, the long turnaround time compromises the applicability of these methods for clinical diagnosis.

The *Taq*-PCR technique has been successfully applied to diagnosis of *Staphylococcus aureus* mastitis in dairy ruminants (20). Although sensitivity and specificity were reasonably high, false-negative results were a limiting factor in applying *Taq*-PCR without further modification to detect *S. aureus nuc* gene in milk. We hypothesized that some unknown *Taq* DNA polymerase inhibitory materials in milk were responsible for the false-negative results associated with the simple DNA extraction procedure.

In the present study, a rapid and simple DNA purification method that incorporated Chelex-100 minimized false-negative PCR results for detecting *S. aureus nuc* gene in milk from clinical and experimental cases of bovine mastitis. The *Taq* DNA polymerase reaction (***Tth* PCR**) conventionally used for PCR was replaced with *Tth* DNA polymerase (32). These modifications enhanced the value of the PCR as a rapid, sensitive, and specific diagnostic tool.

MATERIALS AND METHODS

Animals and Experimental IMI with *S. aureus*

In performing experiments and collecting of samples, we strictly adhered to the humane care of animals as promulgated by USDA, National Institutes of Health, and other agencies and enforced by the University of Illinois Office of Laboratory Animal Resources.

Nine Holstein-Friesian lactating cows were experimentally infected in the right, front quarter by intracisternal administration of approximately 50 cfu of *S. aureus* (Newbould strain 305, ATCC 29740) 12 wk before PCR testing. These intramammary infections persisted for the duration of the trial. No mastitis therapy was given if cows converted from subclinical to clinical mastitis. Some of the cows were low shedders of *S. aureus*, but the overall range of SCC was 800,000 to 9,999,000/ml.

In addition, 100 randomly selected lactating cows at the Dairy Research Farm at the University of Illinois at Urbana-Champaign (**UIUC**), 16 cases of clinical bovine mastitis associated with independent outbreaks at the Veterinary Teaching Hospital, UIUC, and pathogen-spiked milk and PBS samples were used.

Milk Sample Collection

A total of 63 quarter milk samples were collected sequentially from the nine cows experimentally infected with *S. aureus*. An aseptic technique was used, and samples were frozen at –20°C until tested. Three of the samples were discarded because they were not sufficient to run the PCR test, which required 1 ml of starting volume.

Milk samples from the 100 lactating cows were composite samples taken directly from weigh jars in the milking parlor. Milk samples were collected into sterilized tubes and frozen at –20°C until tested.

Milk samples from the 16 clinical cases of bovine mastitis were quarter samples submitted fresh for bacterial culture. After bacteriological analysis, these milk samples were frozen at –20°C until tested by the PCR assay.

Bacteriologic Examination

All milk samples (10 μl) were plated on blood agar medium and incubated aerobically at 37°C for 48 h. Colony identification was by National Mastitis Council Guidelines (29). Plates were quantitatively evaluated for *Staphylococcus aureus* and other bacteria, and the results were recorded as colony-forming units per milliliter of undiluted milk. Any organisms were considered to be *S. aureus* if they were catalase positive, beta hemolytic, and coagulase positive and were agglutinated by specific antisera against *S. aureus*.

Isolation of Bacterial DNA from Milk Samples for Preliminary PCR Assay with *Taq* and *Tth* DNA Polymerase

The overall protocol for target DNA isolation and purification was essentially a modification of the crude bacterial DNA extraction technique by the rapid boil method (38). All procedures were carried out under stringently aseptic conditions. Milk samples were first cultured quantitatively using 10 μl to determine colony-forming units per milliliter of milk. The remaining milk aliquot (1 ml starting volume) was used for preparation of template DNA by combining the simple methods of extensive (five times) washing of the pellet with 1 ml of PBS and centrifuging at 13,600 $\times$ *g* for 10 min at room temperature followed by a rapid boil technique. Amplification was then performed on the crude DNA extracts using the conventional methods employing *Taq* or *Tth* DNA polymerases. One microliter of the template DNA was sufficient for the PCR assay.

Methanol Treatment to Remove Inhibitory Substances from DNA Extracts

Seven crude DNA extracts prepared by the rapid boil method were further treated with methanol (24). This subset of samples consisted of two samples that showed positive results from *Taq*- and *Tth*-PCR, three samples that showed negative results from *Taq*- and *Tth*-PCR, and two samples that showed negative results from *Taq*-PCR and positive results from *Tth*-PCR.

The crude DNA extracts were resuspended in 900 μl of pure laboratory-grade methanol. The suspensions were maintained at 4°C for 48 h and centrifuged at 13,600 $\times$ *g* for 15 min, and the supernatant was discarded. The pellets were resuspended in 900 μl of pure methanol, centrifuged at 13,600 $\times$ *g* for 15 min and the supernatant was discarded. The DNA pellets were desiccated under vacuum. Twenty-two microliters of buffer made up of Tris-HCl, EDTA, and NaCl and 3 μl of NaOH was added to the DNA preparations. The tubes were stored at –20°C until required. One microliter of the DNA extract was used for PCR testing.

DNA Preparation with Chelex-100 (400 Minus Mesh)

Twenty-two crude DNA extracts were treated with Chelex-100 by a modification of the method of Winberg (42). Twelve of the samples had been previously found negative for *Taq*- and *Tth*-PCR, and 10 of the samples had been positive for *Tth*-PCR. The 12 *Taq*- and *Tth*-PCR negative samples included three of the samples treated with methanol.

Briefly, the DNA extracts were resuspended in 1 ml of red blood cell lysis buffer (155 m*M* NH_4Cl, 10 m*M* $KHCO_3$ and 0.1 m*M* EDTA-Na_2, pH 7.4), allowed to stand for 15 min and centrifuged for 10 min at 13,600 $\times$ *g*. The supernatant was decanted and this step was repeated once more. The pellet was resuspended in 1 ml of PBS, vortexed, and centrifuged for 10 min at 13,600 $\times$ *g*. Most of the supernatant was decanted, and the pellet vortexed with residual PBS in the tube. Two hundred microliters of 50% Chelex-100 suspension in distilled water was added to the tube and heated at 95°C for 1 to 2 min. The tubes were centrifuged at 13,600 $\times$ *g* for 30 s. For the PCR assay, the tubes were thawed at room temperature and 10 μl of the supernatant was used to compensate for the 10-fold dilution factor.

Oligonucleotide Primers

The oligonucleotide primers for the *nuc* gene of *S. aureus* were synthesized from previously published sequences (4). These primers have been verified to be *S. aureus nuc* gene specific (4). The sequence of the two primers of 21 and 24 bases, respectively, were as follows:

Primer 1: 5′-GCGATTGATGGTGATACGGTT-3′
Primer 2: 5′-AGCCAAGCCTTGACGAACTAAAGC-3′

PCR Amplification

The PCR amplification was performed in a thermal cycler (MJ Research Inc., Watertown, MA) using *Taq* DNA polymerase (GIBCO BRL, Life technologies, Inc. Gaithersburg, MD) or *Tth* DNA polymerase (Boehringer Mannheim). The PCR reaction mixture was prepared and PCR amplification over 37 cycles was performed essentially as described by Brakstad et al. (4) with only minor modification involving the Chelex steps.

The protocol for *Tth* polymerase PCR was identical to that of *Taq* polymerase. In the case of Chelex-100 treatment, 10 μl of DNA extract was added and the rest of the conditions for PCR were followed as used by Brakstad et al. (4). After amplification, the samples were separated by electrophoresis, stained in ethidium bromide solution, visualized, and photographed with a FOTODYNE transilluminator/camera system (New Berlin, WI).

Tth-PCR Specificity Testing with the Organisms in PBS

Pilot studies were initially carried out with normal milk samples spiked with various organisms in parallel with spiked PBS. For each pure culture (isolate) of *S. aureus*, *Enterobacter cloacae*, *Escherichia coli*, and *Klebsiella pneumoniae*, 1 ml of PBS or normal milk was spiked with 3 cfu of the organism. The specificity of the PCR did not differ for milk and PBS (data not shown). Accordingly, subsequent testing utilized just spiked PBS as a control because both methods were 100% specific.

The specificities of conventional *Tth*-PCR and *Taq*-PCR for detecting major mastitis pathogens in PBS were compared with those achieved after Chelex-100 treatment of DNA extracts. Crude DNA was extracted by the rapid boil method, with Chelex-100 treatment. The tubes were stored at –20°C pending PCR analysis. Ten microliters of supernatant was used for *Tth*-PCR to compensate for the 10-fold dilution factor.

Statistical Analysis

Data were comparatively analyzed using a chi-square goodness-of-fit test. Differences were considered significant if $P < 0.05$ (30).

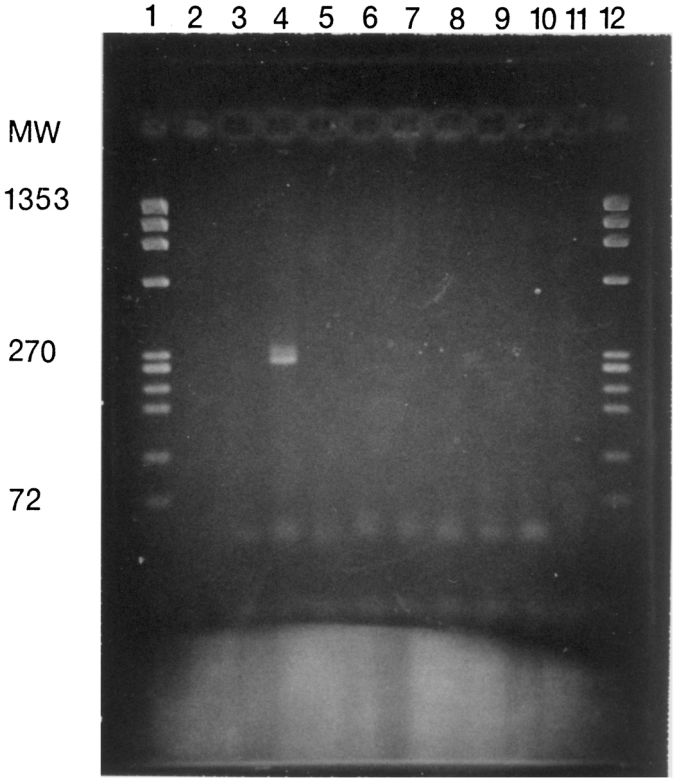

Figure 1. Ethidium bromide stained-agarose gel showing *Tth*-PCR amplification product of *Staphylococcus aureus nuc* gene at 270 bp. Lane 1, PhiX174 DNA marker. Lane 2, blank. Lane 3, negative control containing PCR mixture with distilled water. Lane 4, positive control containing DNA extract from *S. aureus* ATCC 21027. Lanes 5 and 9, milk samples from bovine *S. aureus* mastitis case, perviously positive for *Taq*- and *Tth*-PCR. Lanes 6, 7, and 8, milk samples from bovine *S. aureus* mastitis cases, previously negative for *Taq*- and *Tth*-PCR. Lanes 10 and 11, milk samples from bovine *S. aureus* cases, previously negative for *Tag*-PCR and positive for *Tth*-PCR. Lane 12, PhiX174 DNA marker.

RESULTS

The trial combining the rapid boil method with methanol treatment of DNA extracts gave results unsuitable for clinical use. Two of the 60 milk samples from cows experimentally infected with *S. aureus* were positive by *Taq*- and *Tth*-PCR but did not show any amplification using the *Tth*-PCR after methanol treatment (lanes 5 and 9 in Figure 1).

In comparing *Taq*- and *Tth*-PCR, we applied the *Taq*-PCR method to milk samples taken from cows experimentally inoculated with *S. aureus*. The PCR product was a single DNA band of approximately 270 bp. The *Tth*-PCR method was applied to milk samples from experimental cases of bovine *S. aureus* mastitis, *S. aureus*-free milk samples from cows at the Dairy Research farm, and milk samples from cows with clinical mastitis caused by various pathogens. Amplification was observed only in positive controls during the standardization of the assay (Figure 2).

The sensitivity of bacterial culture for detecting *S. aureus* in mastitic milk samples from the experimentally inoculated cows was 96.7% (Table 1). *Staphylococcus aureus* was cultured from 58 of 60 samples. Milk samples from two cows that were intermittently bacteriologically positive were consistently *Tth*-PCR positive (Figure 3). The latter cows were included in the total of SIA samples and were *Taq* negative.

The *Taq*-PCR on crude DNA from these milk samples yielded a PCR product of 270 bp in 39 of the 60 samples, whereas no amplification was observed in 21 samples (Table 1 and Figures 4 and 5). The two bacteriologically negative samples showed variable *Taq*-PCR positive results (Figure 3).

The sensitivity of *Tth*-PCR on the experimental mastitis samples was 80% (Table 1, Figures 4 and 5). The 48 *Tth*-PCR *S. aureus* positive milk samples included the two bacteriologically negative samples (lanes 5 and

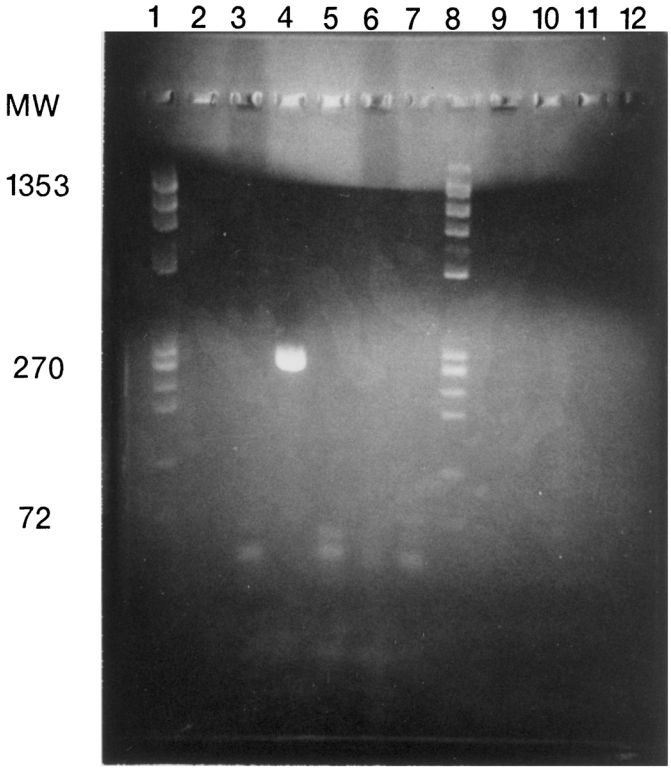

Figure 2. Ethidium bromide stained-agarose gel showing *Tth*-PCR amplification product of *Staphylococcus aureus nuc* gene at 270 bp. Lane 1, PhiX174 DNA marker. Lane 2, blank. Lane 3, negative control containing PCR mixture with distilled water from supernatant of 50% Chelex-100 solution. Lane 4, positive control containing DNA extract from *S. aureus* ATCC 21027. Lane 5, supernatant of Chelex-100 solution. Lane 6, PBS. Lane 7, red-blood cell lysis buffer. Lane 8, PhiX174 DNA marker. Lanes 9 to 12, blank.

Table 1. Comparison of sensitivity of different methods in detection of *Staphylococcus aureus* in milk samples taken from experimentally infected cows.

	Bateriology	*Taq*-PCR	*Tth*-PCR	*Tth*-PCR+Chelex-100
S. aureus Positive	58	39	48	60
S. aureus Negative	2	21	12	0
Sensitivity*	96.7%[a]	65%[b]	80%[c]	100%[a]

[a,b]Different superscripts indicate significant differences in sensitivity percentage ($P < 0.05$).

lane 6 in Figure 3). This result was verified consistently when the *Tth*-PCR test was run in triplicate. Standard *Tth*-PCR was found to be significantly more sensitive than *Taq*-PCR (Table 1; $P < 0.05$).

When crude bacterial DNA was extracted from milk samples by the rapid boil method and the simple DNA purification step with Chelex-100 was performed, results indicated that this protocol was acceptable for detection of *S. aureus* in milk (Table 1). A sensitivity of 100% for detecting *S. aureus* in experimental mastitic milk samples was achieved by combining *Tth*-PCR with Chelex-100 treatment on crude DNA extracts (Table 1). When Chelex-100 treatment was performed on 12 crude DNA extracts which were *Tth*-PCR negative and 10 crude DNA extracts which were *Tth*-PCR positive, the inhibitory effect was removed and positive amplification was obtained (Figures 4 and 5). When Chelex-100 was incorporated into the *Tth*-PCR protocol, statistically significant improvement in sensitivity of the PCR method over traditional *Tth*-PCR was demonstrated (Table 1; $P < 0.05$).

The *Tth*-PCR with Chelex-100 treatment was applied to 100 *S. aureus*-free samples from the Dairy Research Farm, UIUC. None of samples showed an amplified DNA fragment even though pathogens such as *E. coli*, *Streptococcus* spp., nonhemolytic *Staphylococcus* spp., or *Bacillus* spp. were present in some of these samples (Figure 6). Specificity was 100%.

Bacteriological analysis for the milk samples from 16 clinical cases of bovine mastitis revealed *S. aureus* in eight samples, while the other milk samples were *S. aureus* free. None of the samples negative for *S. aureus* were positive by *Tth*-PCR with Chelex-100, but all those positive for *S. aureus* were positive by PCR. Similar levels of sensitivity and specificity were obtained with milk from clinical cases of mastitis (Figure 7).

The specificity of the *Tth*-PCR with Chelex-100 treatment was tested further by using PBS spiked with four major mastitis pathogens (Figure 8). The organisms were *S. aureus*, *Enterobacter cloacae*, *E. coli*, and *Klebsiella pneumoniae*. Amplification was detected from the DNA extract of *S. aureus* and from one in which the four organisms were mixed, while the rest did not show any detectable amplification (Figure 8), indicating 100% sensitivity and specificity.

DISCUSSION

The realization of the full potential of PCR as a diagnostic tool has been constrained by PCR inhibitors found in various biological specimens. Nevertheless, PCR has been successfully applied in other research fields such as genomic DNA sequencing, chromosomal rearrangements, and high-efficiency cloning of genomic sequences (22, 25, 34, 43, 44). In the present study, a rapid and highly efficient method to avoid the PCR inhibitory effects of substances in DNA extracts from milk was developed. This method was able to detect *S. aureus* in milk from cows with experimentally induced *S. aureus* mastitis and was 100% sensitive and specific when applied to milk samples from *S. aureus* free cows, milk from cows with mastitis, milk from chronic mastitis cases, and pathogen-spiked PBS samples.

The *Taq* DNA polymerase and *Tth* DNA polymerase were compared for ability to detect *S. aureus nuc* gene in milk samples. *Taq*-PCR was performed on 60 milk samples from cows experimentally infected with *S. aureus* after the rapid boil method to extract DNA from milk samples. Without further treatment on crude DNA extracts, the sensitivity for detecting *S. aureus nuc* gene was only 65%, indicating that *Taq*-PCR is impaired by inhibitors in milk. Other investigators have reported that, due to the PCR interfering effects of milk, the *Taq*-PCR detection limit in milk or dairy products is much lower than that in PBS or in water (13, 32, 39, 41). *Taq* DNA polymerase inhibitors previously have been demonstrated in a variety of clinical and nonclinical samples. Blood, mucus, urine, sperm, and fecal material are some of the body fluids and products that have been shown to inhibit the enzyme (4, 19, 21, 26, 31, 40). Furthermore, substances such as heparin and phenol, and certain concentrations of KCl, Mg^{2+}, ammonium chloride, urea, dimethyl sulfoxide, dimethylformamide, formamide, polyamines, plant polysaccharides, calcium alginate, and SDS have inhibitory effects on *Taq* DNA polymerase activity (3, 9, 11, 12, 17, 40). In addition, Hoppe et al. (16) found that many dyes, including the traditional DNA loading dyes, bromophenol blue and xylene cyanol, completely inhibit *Taq*-PCR, even at low concentrations. Therefore, the necessity of replacing *Taq* DNA polymerase with another thermo-

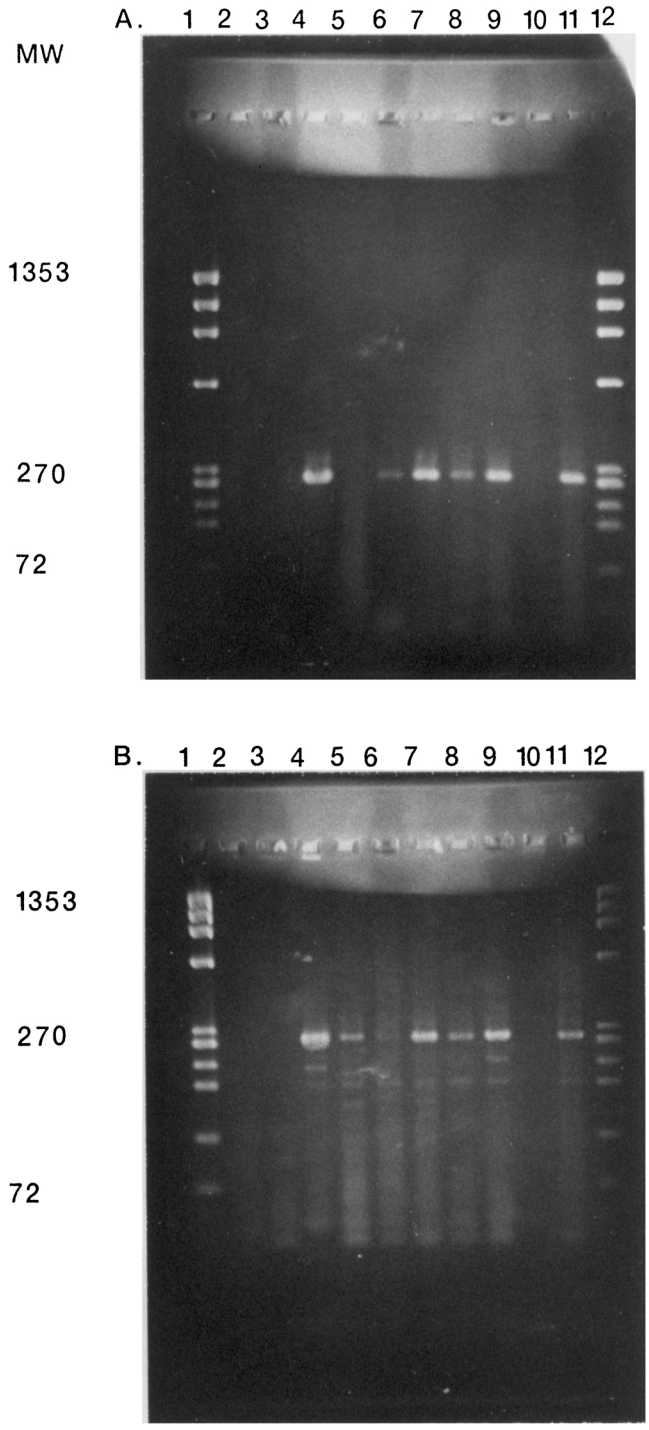

Figure 3. A, *Taq*-PCR and B, *Tth*-PCR. Ethidium bromide stained-agarose gel showing amplification product of *Staphylococcus aureus nuc* gene at 270 bp. Lane 1, PhiX174 DNA marker. Lane 2, blank. Lane 3, negative control containing PCR mixture with distilled water. Lane 4, positive control containing DNA extract from *S. aureus* ATCC 21027. Lanes 5 to 11, milk samples taken from cows experimentally infected with *S. aureus*. Each lane represents results from experimental category A or B of each target DNA preparation. Note Lanes 5 and 6 containing milk samples that were culture negative but found to be PCR positive. Lane 12, PhiX174 DNA marker.

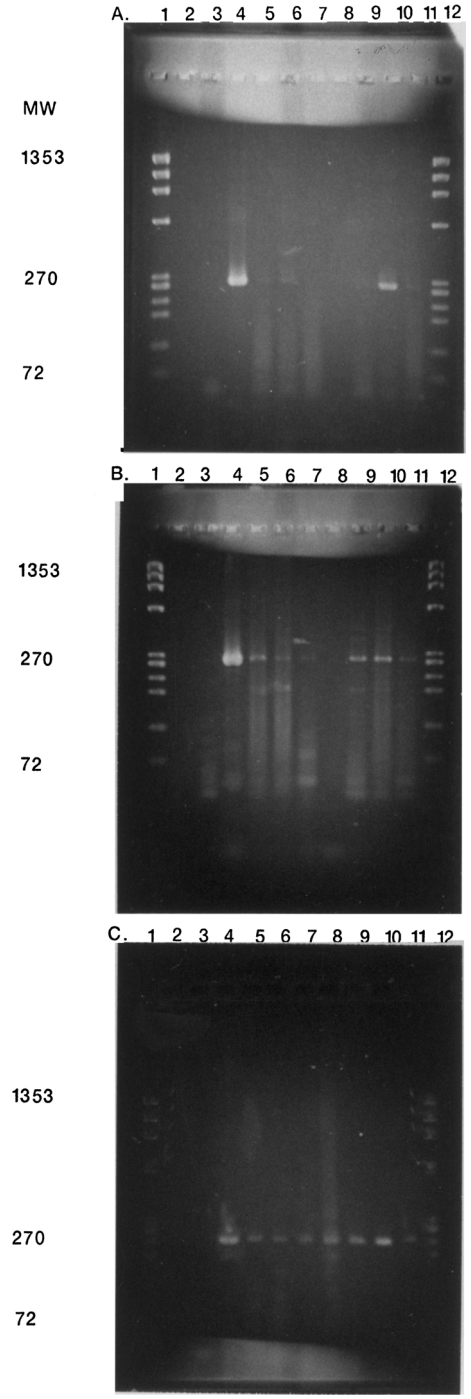

Figure 4. Comparisons of results of 3 PCR methods for detecting *Staphylococcus aureus nuc* gene in milk from cows experimentally infected with *S. aureus*. A, *Taq*-PCR, B, *Tth*-PCR, and C, *Tth*-PCR after Chelex-100 treatment. Ethidium bromide stained-agarose gel showing amplification product of *Staphylococcus aureus nuc* gene at 270 bp. Lane 1, PhiX174 DNA marker. Lane 2, blank. Lane 3, negative control containing PCR mixture with distilled water. Lane 4, positive control containing DNA extract from *S. aureus* ATCC 21027. Lanes 5 to 11, milk samples taken from cows experimentally infected with *S. aureus*. Each lane represents results from experimental category A, B, or D of each target DNA preparation. Lane 12, PhiX174 DNA marker.

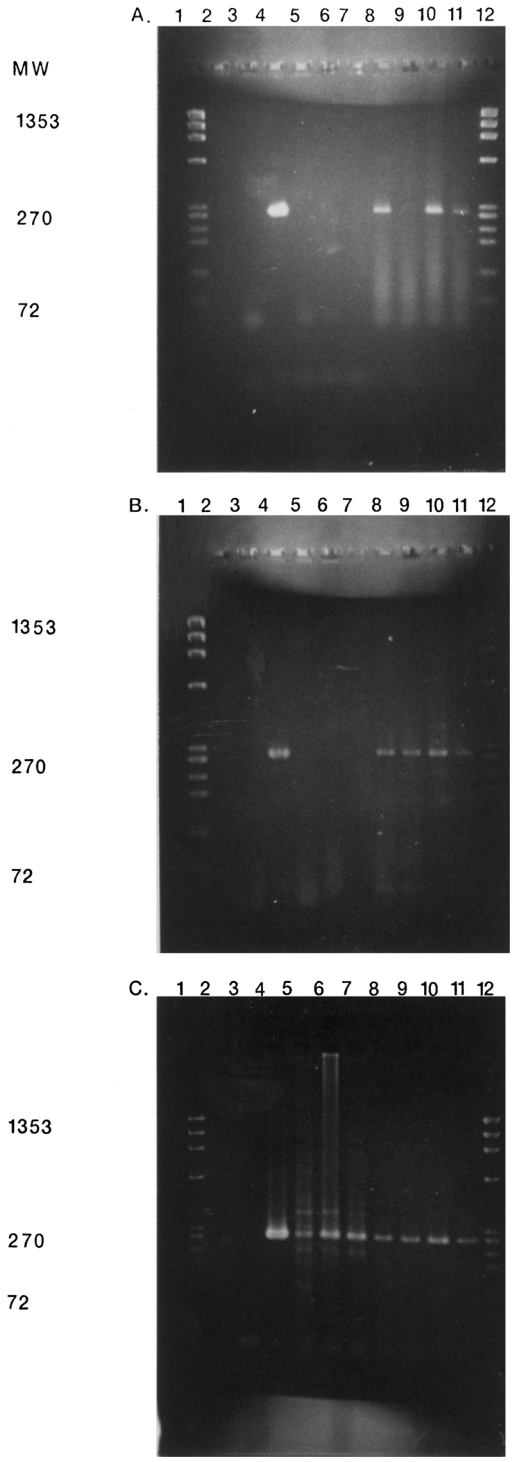

Figure 5. A, *Taq*-PCR, B, *Tth*-PCR, and C, *Tth*-PCR after Chelex-100 treatment. Ethidium bromide stained-agarose gel showing amplification product of *Staphylococcus aureus nuc* gene at 270 bp. Lane 1, PhiX174 DNA marker. Lane 2, blank. Lane 3, negative control containing PCR mixture with distilled water. Lane 4, positive control containing DNA extract from *S. aureus* ATCC 21027. Lanes 5 to 11, milk samples taken from cows experimentally infected with *S. aureus*. Each lane represents results from experimental category A, B, or D of each target DNA preparation. Lane 12, PhiX174 DNA marker.

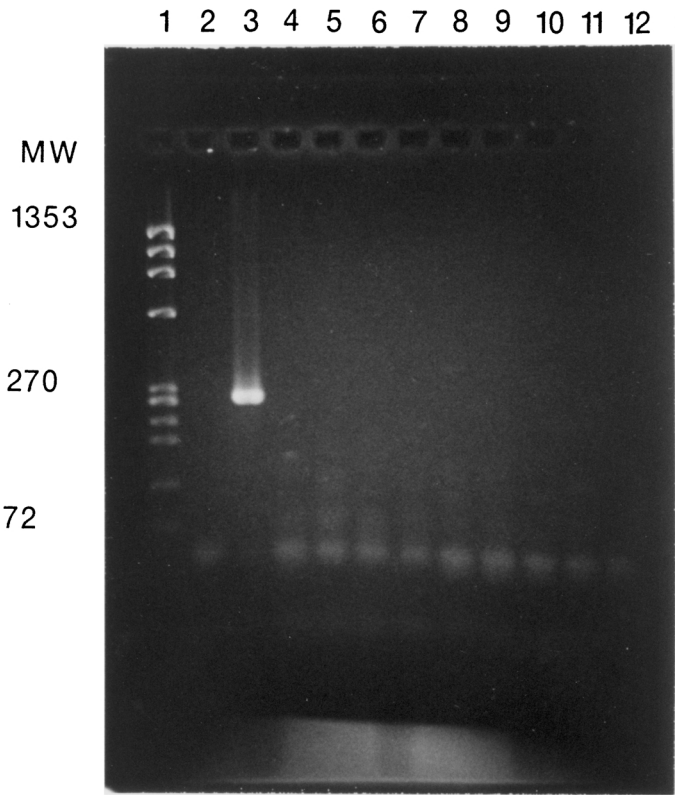

Figure 6. Ethidium bromide stained-agarose gel showing *Tth*-PCR amplification product of *Staphylococcus aureus nuc* gene at 270 bp. Lane 1, PhiX174 DNA marker. Lane 2, negative control containing PCR mixture with distilled water from supernatant of 50% Chelex-100 solution. Lane 3, positive control containing DNA extract from *S. aureus* ATCC 21027. Lanes 4 to 12, milk samples from cows not infected with *S. aureus*.

stable DNA polymerase to improve PCR sensitivity for detecting *S. aureus nuc* gene in bovine milk was evident.

In our study, *Tth* DNA polymerase was selected as a substitute for *Taq* DNA polymerase. The PCR sensitivity for detecting *S. aureus* in milk from inoculated cows was raised to 80% when *Tth* DNA polymerase was used, compared with 65% obtained with *Taq* DNA polymerase. Other investigators have shown similar improvement in sensitivity using *Tth* DNA polymerase. According to Panaccio and Lew (31), while *Taq* DNA polymerase was totally inhibited by the presence of as little as 1 μl of blood, *Tth* DNA polymerase, from *Thermus thermophilus* HB8 was able to successfully amplify a specific 1.3-kb target sequence in a PCR reaction mix containing up to 4% (vol/vol) blood. In contrast to observations in which *Taq* and Stoffel fragment were inhibited by aqueous and vitreous fluids, the *Tth* DNA polymerase reaction was completely resistant to the inhibitory effects of 20 μl of vitreous fluid (40). Furthermore, Katcher and Schwartz (18) showed that *Tth* DNA

polymerase can function normally in the presence of 5% phenol, whereas traces of phenol completely inactivated *Taq* polymerase.

Interestingly, our data have shown that *Taq*-PCR and *Tth*-PCR could detect and amplify target DNA in two bacteriologically negative milk samples from *S. aureus* infected cows. A possible explanation is that the PCR method may have detected organisms present in concentrations too low to be detected by culture. Because *S. aureus* is shed intermittently and can be walled off in the mastitic gland, the bacteriological culture method might show false-negative results while the PCR tests could detect and amplify residual *S. aureus* DNA or sequestered organisms (Figure 3, lanes 5 and 6). The true positivity of the PCR protocol was also consistently confirmed by its correlation with elevated SCC in low shedders with chronic mastitis. It is also plausible that disproportionately high concentrations of host DNA that are invariably associated with high SCC may nonspecifically interfere with specific amplification of the DNA of low numbers of the pathogen (7). In triplicate samples, *Tth*-PCR showed consistent amplification from the two bacteriologically negative samples while *Taq*-PCR did not, indicating that *Tth*-PCR is more sensitive than *Taq*-PCR.

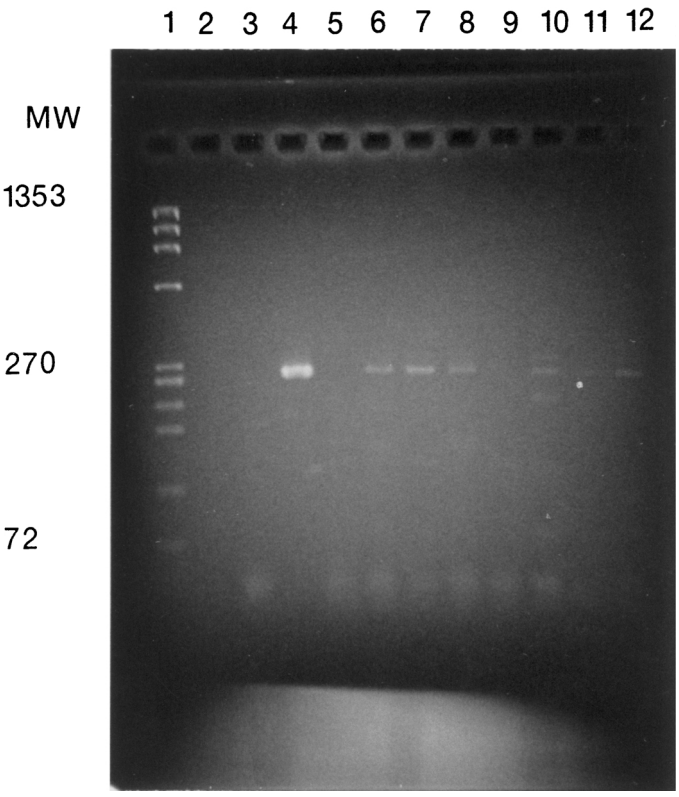

Figure 7. Ethidium bromide stained-agarose gel showing *Tth*-PCR amplification product of *Staphylococcus aureus nuc* gene at 270 bp. Lane 1, PhiX174 DNA marker. Lane 2, blank. Lane 3, negative control containing PCR mixture with distilled water from supernatant of 50% Chelex-100 solution. Lane 4, positive control containing DNA extracat from *S. aureus* ATCC 21027. Lanes 5 and 9, milk samples from clinical case of bovine mastitis caused by microorganisms (*Bacillus* species, yeast) other than *S. aureus*. Lanes 6, 7, 8, 10, 11, and 12, milk samples from clinical case of bovine *S. aureus* mastitis.

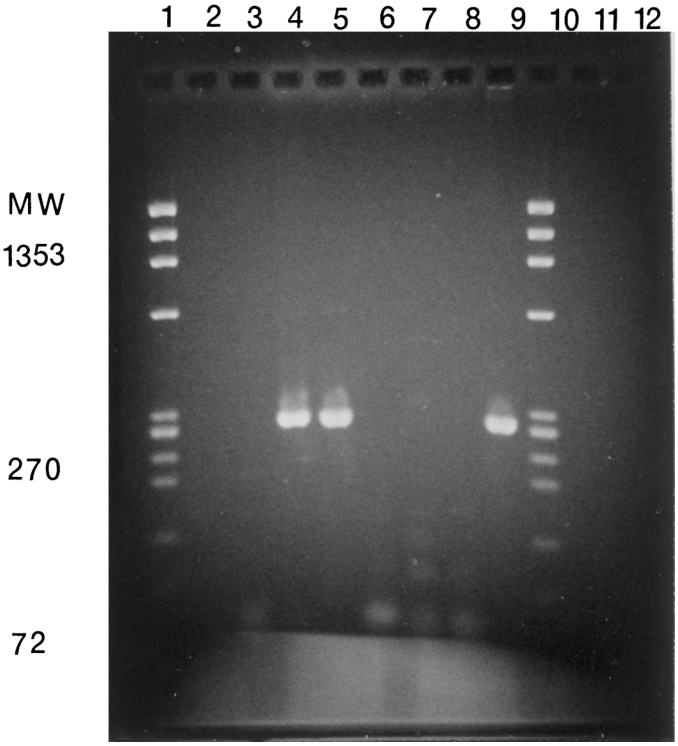

Figure 8. Ethidium bromide stained-agarose gel showing *Tth*-PCR amplification product of *Staphylococcus aureus nuc* gene at 270 bp. Lane 1, PhiX174 DNA marker. Lane 2, blank. Lane 3, negative control containing PCR mixture with distilled water from supernatant of 50% Chelex-100 solution. Lane 4, positive control containing DNA extract from *S. aureus* ATCC 21027. Lane 5, DNA extract from clinically isolated *S. aureus*. Lane 6, DNA extract from clinically isolated *Enterobacter cloacae*. Lane 7, DNA extract from clinically isolated *Escherichia coli*. Lane 8, DNA extract from clinically isolated *Klebsiella pneumoniae*. Lane 9, mixed DNA extract from the organisms (*S. aureus*, *E. cloacae*, *E. coli*, *K. pneumoniae*). Lane 10, PhiX174 DNA marker. Lanes 11 and 12, blank.

Because simple replacement of *Taq* DNA polymerase did not abrogate PCR inhibitory effects in milk samples, further modification of the PCR procedure was necessary to improve *Tth*-PCR sensitivity and to apply *Tth*-PCR as a diagnostic tool using milk samples.

It was observed in a previous study that increasing the number of PBS washes from two to five on the *S. aureus* DNA pellet obtained from the rapid boil method increased the sensitivity of the *Taq*-PCR method for detecting *S. aureus* in mastitic milk samples (20). However, the results from our study show that stringent

washing combined with the rapid boil method did not completely remove *Taq*-PCR inhibitory effects from milk samples. A previous study demonstrated that milk samples that showed no *Taq*-PCR amplification after five washes did not amplify even after five more washes with PBS, suggesting that existence of some PCR-inhibitory effects in milk may not be removed by washing alone. Furthermore, inhibitory substances in milk samples inhibited *Tth*-PCR to the extent that only 80% sensitivity was achieved. Our data indicate that milk, unlike other specimens such as blood and vitreous fluid (31, 40), may inhibit *Tth*-PCR. However, *Tth* appears to be less sensitive to inhibitors than *Taq*. This aspect warrants further studies.

Methanol treatment of crude DNA extracts has been successfully used to improve results of *Taq*-PCR on blood samples (24). However, in our study methanol treatment of crude DNA extracts from the rapid boil method was not suitable for detecting *S. aureus nuc* gene in bovine milk using *Tth*-PCR. Two milk samples that showed positive results by *Taq*- and *Tth*-PCR did not amplify after methanol treatment on the crude DNA extracts. This contrasts with data on methanol treatment of DNA from other types of clinical specimens (18, 40).

Chelex-100 treatment on crude DNA extracts successfully removed inhibitory effects from milk samples. Chelex-100 is a negatively charged resin in neutral and alkaline conditions and is insoluble in water. Singer-Sam et al. (35) used Chelex-100 as a means of increasing the signal from the PCR amplification of small amounts of DNA released from tissue culture cells that had been boiled. Chelex-100 can chelate a large number of divalent ions that may be donated by the sample, and the Chelex beads can be easily removed so that they will not interfere with subsequent PCR amplifications that require Mg^{2+} (37). Our pilot studies indicate that *Tth*-PCR could amplify target DNA even if high concentrations of major metal ions (0.01 m*M* $FeCl_3$ or $CaCl^2$) were present in the PCR reaction tubes (data not shown). In our study, the treatment of crude DNA extracts with Chelex-100 appeared suitable for *Tth*-PCR detection of *S. aureus nuc* gene in milk and raised the sensitivity to 100%. Walsh et al. (37) had similar success using Chelex-100 treatment for *Taq*-PCR with blood samples from crime scenes. More importantly, since incorporation of Chelex-100 treatment into *Tth*-PCR enhances PCR sensitivity for milk samples, this method has potential for routine diagnostic use. When Chelex-100 treatment was used in two bacteriologically positive *S. aureus* milk samples that showed negative results by methanol treatment and by *Taq*- and *Tth*-PCR yielded amplification products (Figures 3 to 5). To achieve adequate turnround time with PCR, this simple modification of the boiling method is appropriate in diagnostic application where simple, fast, and accurate methods are preferred over the time-consuming DNA purification methods.

The specificity of PCR is, in part, determined by the primers used and the primers that flank the *nuc* gene of *S. aureus* have been verified by Southern blot analysis of the amplified DNA confirming the origin of the DNA fragment (4). Various mastitis-causing organisms were tested by *Taq*-PCR with these primers, and only *S. aureus* strains were amplified (4). In our study, using milk from cows with clinical or subclinical mastitis or normal milk no amplification was observed when non-*S. aureus* bacteria or no bacteria were present. The specificity was 100% and was tested against *S. aureus* isolates from independent mastitis outbreaks or experimental situations. Additional field tests are warranted before universal adoption of the protocol for routine mastitis testing.

Perhaps the most important contribution of our data is the feasibility of *Tth*-PCR for routine diagnosis on milk samples. This modified *Tth*-PCR protocol may have applications for detecting food contaminants such as *Listeria*, *Brucella*, and bovine immunodeficiency virus (13, 28, 32, 39, 41) in milk. Our data have demonstrated that the addition of Chelex-100 to the *Tth*-PCR protocol significantly enhances sensitivity without compromising the specificity of the assay. The exact mechanism of action of Chelex-100, especially when applied to milk samples, remains to be determined.

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